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Effects of sulfhydryl reagents on the Cys^{65} mutant of the transposon Tn10-encoded metal-tetracycline/H$^+$ antiporter of *Escherichia coli*

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The Cys^{65} mutant of the Tn10-encoded metal-tetracycline/H$^+$ antiporter is the only one which is inactivated by sulfhydryl reagents among the Cys mutants of the putative loop$_{2-3}$ region [Yamaguchi, A. et al. (1992) J. Biol. Chem. 267, 19155–19162]. The tetracycline transport activity of the Cys^{65} mutant was completely abolished by *N*-ethylmaleimide; however, methyl methanethiosulfonate only abolished 45% of the activity, even in the presence of an excess of the reagent. Since *N*-ethylmaleimide did not further inactivate the methyl methanethiosulfonate-treated antiporter, it is clear that the modified antiporter molecule with a small substituent, a thiomethyl group, had significant but lower activity than the unmodified antiporter. The binding of [^{14}C]*N*-ethylmaleimide to the Cys^{65} mutant was inhibited in the predsence of tetracycline. These findings indicate that position 65 is close to the site of the interaction with the substrate and the modification of the side chain at this position caused steric hindrance as to substrate translocation.

Tetracycline; Antiporter; Tetracycline/H$^+$ antiporter; Sulfhydryl reagent; Antibiotic resistance; Site-directed mutagenesis

1. INTRODUCTION

The transposon Tn10-encoded metal-tetracycline/H$^+$ antiporter (Tn10-TetA) is one of the class B tetracycline resistance determinants and confers the highest resistance among the bacterial tetracycline resistance determinants [1]. The sequences of the class A, B, and C TetA proteins are highly homologous [2], and, on the basis of the hydropathy profiles, a secondary structure model composed of 12 membrane-spanning α-helices and 11 interhelix loops was proposed [3,4]. The putative structure was supported by the results of protease digestion [3] and antibody binding [3,5], and the finding that fusions of the TetA protein to alkaline phosphatase [6] have both their N- and C-termini on the cytoplasmic side [3,5,6].

The putative loop$_{2-3}$ of Tn10-TetA, which was assumed to be located on the cytoplasmic surface and to be composed of 10 residues [4], is especially homologous not only in the class A, B, and C TetA proteins [2], but also in the bacterial drug efflux proteins [7,8] and other secondary transporters, including sugar/H$^+$ symporters and glucose transporters [9]. Loop$_{2-3}$ was originally predicted to be a tetracycline binding site [10]. In a previous paper, we showed that the negative charge of Asp^{66} at the middle of this loop is essential for the tetracycline transport function [11]. Since the substrate transported is a monocationic chelation complex [12], it is possible that Asp^{66} is the residue that first interacts with the substrate. Ser^{65} itself is not important for the transport function, yet the Cys^{65} mutant of the Tn10-TetA protein was completely inactivated by *N*-ethylmaleimide (NEM) [11]. The wild type TetA protein has only one cysteine residue, at position 377, but its activity is not affected by NEM [11]. Surprisingly, the Cys^{65} mutant was the only Cys mutant of which the transport activity was abolished by NEM among the Cys mutants of the loop$_{2-3}$ region [4].

In this work, we found that the degree of inactivation of the Cys^{65} mutant by sulfhydryl reagents depends on the volume of the substituent, and the modification of Cys^{65} by [^{14}C]NEM was inhibited by the presence of excess tetracycline. These observations supported the hypothesis that Asp^{66} is the residue that first interacts with a monocationic substrate and the inactivation of the Cys^{65} mutant by sulfhydryl reagents was due to the neighboring bulky substituent exerting steric hindrance on the substrate–Asp^{66} interaction.

2. MATERIALS AND METHODS

2.1. *Materials*

N-Ethyl[2,3-^{14}C]maleimide (333 MBq/mmol) and [7-^{3}H(*N*)]tetracycline (23.7 GBq/mmol) were purchased from Amersham and DuPont-New England Nuclear, respectively. Methyl methanethiosulfonate was purchased from Aldrich Chemical Co. All other materials were of reagent grade and obtained from commercial sources.

2.2. *Plasmids*

pLGT2 is a low-copy-number plasmid containing the wild type Tn10-*tet*A and *tet*R genes [13]. pLGS65C is a derivative of pLGT2

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encoding the Cys^{65} mutant *tet*A gene which was constructed by site-directed mutagenesis [11].

2.3. *Preparation of inverted membrane vesicles and assay of transport*

Inverted membrane vesicles were prepared from *Escherichia coli* W3104 [14] cells carrying pLGT2 or pLGS65C as described previously [15]. [^{3}H]Tetracycline uptake by inverted membrane vesicles was assayed as described previously [12] in the presence of 50 μM $CoCl_2$, 10 μM [^{3}H]tetracycline and 2.5 mM NADH in 50 mM MOPS–KOH buffer (pH 7.0) containing 0.1 M KCl unless otherwise stated. [^{3}H]Tetracycline was diluted 5 times with unlabeled tetracycline just before use.

2.4. *Assaying of binding of [^{14}C]N-ethylmaleimide to TetA proteins*

Inverted membrane vesicles (0.5 mg protein) were incubated in 100 μl of 50 mM MOPS–KOH buffer (pH 7.0) containing 0.1 M KCl, 5 mM $MgSO_4$ and 0.5 mM [^{14}C]NEM at 30°C for 1 min in the presence or absence of 1 mM tetracycline. Immediately after the incubation, the vesicles were diluted with 800 μl of 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl and excess unlabeled NEM (10 mM) to stop the binding. Then the vesicles were precipitated by ultracentrifugation at 200,000 × *g* for 30 min at 4°C with a Beckman ultracentrifuge, Optima TL. The resultant precipitate was solubilized in 200 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl, 1% Triton X-100, 0.1% SDS and 5 mM unlabeled NEM by brief sonication with a Branson bath sonifier. Insoluble substances were removed by ultracentrifugation at 200,000 × *g* for 30 min at 4°C. The resultant supernatant was mixed with 15 μl of anti-TetA-carboxyl-terminal antiserum [5] in an Eppendorf tube, followed by incubation at room temperature for 1 h with shaking. Then 120 μl of a Pansorbin *S. aureus* cell suspension (Carbiochem) [16] was added to the mixture and the incubation was continued for a further 1 h. The immunoprecipitate was collected by centrifugation at 1,000 × *g* for 10 min at room temperature, and then washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl, 1% Triton X-100 and 0.1% SDS. SDS gel electrophoresis of the precipitate was performed and the radioactive band was visualized by autoradiography.

3. RESULTS AND DISCUSSION

3.1. *Inactivation of the Cys^{65} mutant TetA protein by N-ethylmaleimide and methyl methanethiosulfonate*

Inverted membrane vesicles were prepared from *E. coli* W3104 cells carrying pLGT2 or pLGS65C. Ten μl of the vesicle suspension (35 μg protein) in 50 mM MOPS–KOH buffer (pH 7.0) containing 0.1 M KCl was mixed with 0.5 μl of various concentrations of NEM or methyl methanethiosulfonate (MMTS), followed by incubation for 29 min at 30°C. Then, 0.5 μl of 250 mM NADH was added and the incubation was continued for a further 1 min. Tetracycline uptake was started by the addition of 40 μl of an assay mixture comprising 12.5 μM [^{3}H]tetracycline (final conc. 10 μM), 62.5 μM $CoCl_2$ (final conc. 50 μM) and 0.1 M KCl in 50 mM MOPS–KOH buffer (pH 7.0). After 30 s incubation at 30°C, 2 ml of a wash buffer comprising 0.15 M LiCl in 5 mM MOPS–KOH (pH 7.0) was added, and then the mixture was rapidly filtered through a nitrocellulose filter (24 mm diameter; pore size, 0.45 μm). The filter was washed twice with 2 ml of the wash buffer and then the radioactivity on it was determined with a liquid scintillation counter.

Active transport activity was calculated by subtracting the initial 30 s uptake of [^{3}H]tetracycline in the absence of NADH from that in the presence of NADH. The residual activity, expressed as a percentage, was determined by dividing the residual active transport activity by the activity of the vesicles incubated in the absence of a sulfhydryl reagent. As shown in Fig. 1, 1 mM NEM caused complete inactivation of the Cys^{65} mutant vesicles, while the wild type vesicles were unaffected by 5 mM NEM (data not shown). On the other hand, only 45% of the activity of the Cys^{65} mutant vesicles was abolished by excess MMTS (5 mM) (Fig. 1). This incomplete inactivation is not due to incomplete modification of the Cys^{65} mutant TetA protein by MMTS, because when the vesicles were at first incubated with 2 mM MMTS for 5 min followed by incubation with various concentrations of NEM for 30 min, the degree of the inactivation did not increase any further than the level of inactivation with MMTS alone. Thus, it is clear that all of the Cys^{65} mutant TetA molecules were modified with a thiomethyl substituent after 5 min incubation with MMTS and each thiomethyl modified TetA molecule is about 55% active compared with an unmodified one.

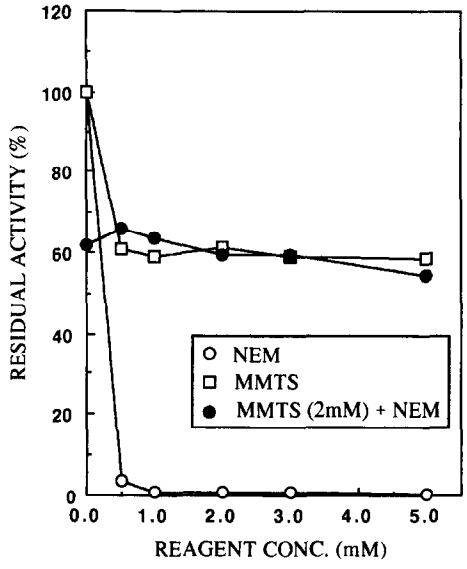

Fig. 1. Inactivation of tetracycline transport activity of Cys^{65} mutant inverted membrane vesicles by *N*-ethylmaleimide and methyl methanethiosulfonate. The inactivation and the transport assay were performed as described in section 2. Open circles and open squares represent the residual activity after treatment with NEM and MMTS for 30 min, respectively. Closed circles represent the activity after sequential treatment with 2 mM MMTS for 5 min and then the indicated concentrations of NEM for 30 min.

In a previous paper [11], we reported that the degree of inactivation of the Cys^{65} mutant by MMTS was about 60%. In the present work, the degree of inactivation was less than that in the previous work. One reason for this is that the level of inactivation was overestimated in the previous work because the residual activity was normalized as to the activity before incubation. The

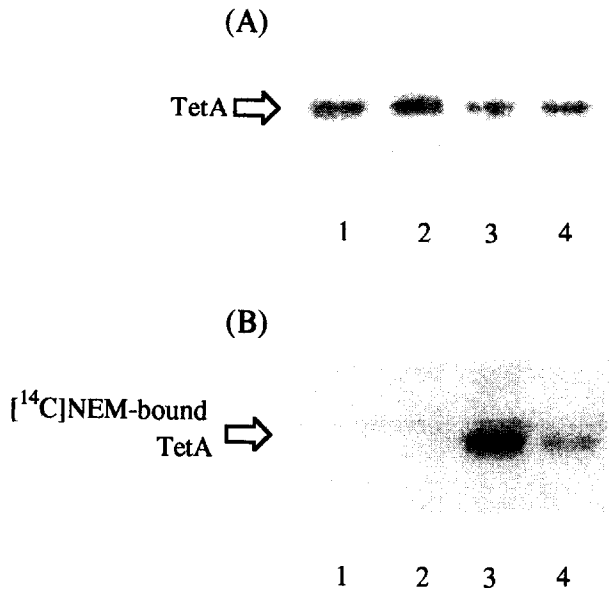

Fig. 2. Binding of [^{14}C]NEM to TetA proteins. Vesicles containing wild type TetA (lanes 1 and 2) or Cys^{65} mutant TetA (lanes 3 and 4) were incubated with [^{14}C]NEM in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM tetracycline. After solubilization of the vesicles, the TetA proteins were immunoprecipitated as described in section 2, followed by SDS polyacrylamide gel electrophoresis. (A) Coomassie brilliant blue staining. (B) Autoradiography.

incubation of the vesicles without a sulfhydryl reagent caused a little decrease in the activity. The residual activity in the present work was normalized as to the activity of vesicles incubated for the same period in the absence of a reagent. However, such normalization could not explain all the discrepancy between the results. Anyway, the degree of inactivation in the present work was highly reproducible and the discrepancy does not change the conclusion.

Sequential treatment with MMTS and NEM solved the outstanding problem in the previous work of whether the incomplete inactivation by MMTS was due to incomplete modification of the Cys^{65} mutant by MMTS or because each molecule of the thiomethyl modified Cys^{65} TetA protein had lower activity than the unmodified one. The latter is the case. This suggests that the inactivation is due to simple steric hindrance without protein denaturation.

3.2. *Inhibition of [^{14}C]N-ethylmaleimide binding to the Cys^{65} mutant TetA by tetracycline*

As shown in Fig. 2, when the Cys^{65} mutant vesicles were incubated with 0.5 mM [^{14}C]NEM for 1 min in the absence of tetracycline, a radioactive band corresponding to the TetA protein was detected on autoradiography. In contrast, the wild type vesicles showed no such radioactive band. Thus, it is clear that the radioactive band reflects the modification of Cys^{65} by [^{14}C]NEM. When the Cys^{65} mutant vesicles were incubated with 0.5 mM [^{14}C]NEM in the presence of 1 mM tetracycline for 1 min, the density of the radioactive band decreased substantially (Fig. 2). Since there was no significant difference in the amounts of the TetA proteins visualized on Coomassie brilliant blue staining, the decrease in the radioactivity indicates the inhibition of NEM binding by tetracycline.

We previously reported that the inactivation of the Cys^{65} mutant by NEM was not affected by the presence of 50 μM tetracycline [11]. In that experiment, the concentration of tetracycline was 20-fold lower than that of NEM, and the vesicles were treated with NEM for 5 min, which was 5 times longer than in the present work. Therefore, it is concluded that the short-time modification by NEM was inhibited by tetracycline at a concentration higher than that of NEM. The K_m value of the antiporter as to tetracycline is around 20 μM [11,13]. The concentration of tetracycline required for inhibition of the NEM modification was considerably higher than the K_m value. Thus, it seems unlikely that the inhibition reflects the binding of tetracycline to the substrate recognition site of the antiporter, although it is certain that there is some interaction between tetracycline and a site near Cys^{65} leading to competitive inhibition of Cys^{65} modification by NEM.

The negative charge of Asp^{66} is essential for the tetracycline transport function and is predicted to interact with a monocationic divalent–cation–tetracycline chelation complex [11]. It is likely that this interaction inhibits the Cys^{65} modification by NEM (Fig. 3). These observations supported the hypothesis that the loop$_{2-3}$ region is a gate at the entrance of the predicted tetracycline transport channel and that it first interacts with a chelation substrate. The substrate recognition site may be located in a deep transmembrane channel.

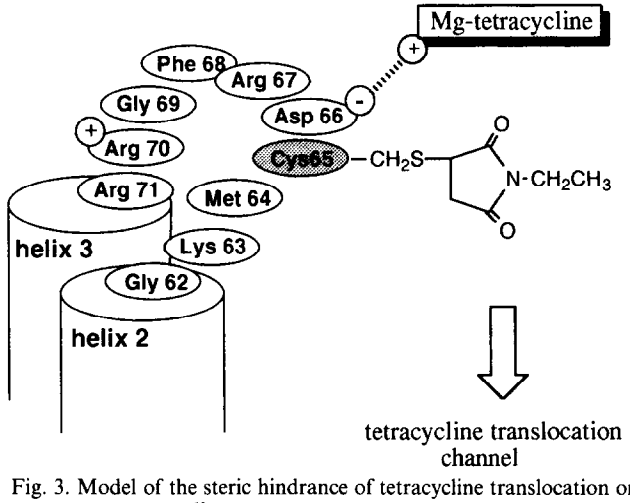

Fig. 3. Model of the steric hindrance of tetracycline translocation on modification of Cys^{65} with *N*-ethylmaleimide. The residues constituting the putative loop$_{2-3}$ of the Cys^{65} mutant TetA protein are depicted as ovals. A positive charge of a divalent cation–tetracycline complex is predicted to first interact with the negative charge of Asp^{66}, and then the complex molecule enters the putative substrate translocation channel. A bulky substituent of Cys^{65} may be located at the entrance of the channel and thus hinder the tetracycline translocation.

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REFERENCES

[1] Mendez, B., Tachibana, C. and Levy, S.B. (1980) Plasmid 3, 99–108.
[2] Waters, S.H., Rogowsky, P., Grinsted, J., Altenbuchner, J. and Schmitt, R. (1983) Nucleic Acids Res. 11, 6089–6105.
[3] Eckert, B. and Beck, C.F. (1989) J. Biol. Chem. 264, 11663–11670.
[4] Yamaguchi, A., Someya, Y. and Sawai, T. (1992) J. Biol. Chem. 267, 19155–19162.
[5] Yamaguchi, A., Adachi, K. and Sawai, T. (1990) FEBS Lett. 265, 17–19.
[6] Allard, J.D. and Bertrand, K.P. (1992) J. Biol. Chem. 267, 17809–17819.
[7] Yoshida, H., Bogaki, M., Ubukata, K. and Konno, M. (1990) J. Bacteriol. 172, 6942–6949.
[8] Neyfakh, A.A., Bidenko, V.E. and Chen, L.B. (1991) Proc. Natl. Acad. Sci. USA 88, 4781–4785.
[9] Henderson, P.J.F. (1990) J. Bioenerg. Biomembr. 22, 525–569.
[10] Chopra, I. (1986) J. Antimicrob. Chemother. 18, Suppl. C, 51–56.
[11] Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T. and Sawai, T. (1990) J. Biol. Chem. 265, 15525–15530.
[12] Yamaguchi, A., Udagawa, T. and Sawai, T. (1990) J. Biol. Chem. 265, 4809–4813.
[13] Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M. and Sawai, T. (1992) J. Biol. Chem. 267, 7490–7498.
[14] Yamamoto, T., Tanaka, M., Nohara, C., Fukunaga, Y. and Yamagishi, S. (1981) J. Bacteriol. 145, 808–813.
[15] Yamaguchi, Y., Adachi, K., Akasaka, T., Ono, N. and Sawai, T. (1991) J. Biol. Chem. 266, 6045–6051.
[16] Philipson, L., Anderson, P., Olshevsky, U., Weinberg, R. and Baltimore, D. (1978) Cell 13, 189–199.